The Commensal *Streptococcus salivarius* K12 Downregulates the Innate Immune Responses of Human Epithelial Cells and Promotes Host-Microbe Homeostasis[∇][†]

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Streptococcus salivarius **is an early colonizer of human oral and nasopharyngeal epithelia, and strain K12 has reported probiotic effects. An emerging paradigm indicates that commensal bacteria downregulate immune responses through the action on NF-κB signaling pathways, but additional mechanisms underlying probiotic actions are not well understood. Our objective here was to identify host genes specifically targeted by K12 by comparing their responses with responses elicited by pathogens and to determine if** *S. salivarius* **modulates epithelial cell immune responses. RNA was extracted from human bronchial epithelial cells (16HBE14O- cells) cocultured with K12 or bacterial pathogens. cDNA was hybridized to a human 21K oligonucleotide-based array. Data were analyzed using ArrayPipe, InnateDB, PANTHER, and oPOSSUM. Interleukin 8 (IL-8) and growth-regulated oncogene alpha (Groα) secretion were determined by enzyme-linked immunosorbent assay. It was demonstrated that** *S. salivarius* **K12 specifically altered the expression of 565 host genes, particularly those involved in multiple innate defense pathways, general epithelial cell function and homeostasis, cytoskeletal remodeling, cell development and migration, and signaling pathways. It inhibited baseline IL-8 secretion and IL-8 responses to LL-37,** *Pseudomonas aeruginosa,* **and flagellin in epithelial cells and attenuated Groα secretion in response to flagellin. Immunosuppression was coincident with the inhibition of activation of the NF-κB pathway. Thus, the commensal and probiotic behaviors of** *S. salivarius* **K12 are proposed to be due to the organism (i) eliciting no proinflammatory response, (ii) stimulating an anti-inflammatory response, and (iii) modulating genes associated with adhesion to the epithelial layer and homeostasis.** *S. salivarius* **K12 might thereby ensure that it is tolerated by the host and maintained on the epithelial surface while actively protecting the host from inflammation and apoptosis induced by pathogens.**

Bacteria within the resident communities that colonize mucosal sites outnumber cells of the human body by 10-fold. Such populations are diverse as well as numerous; for example, around 700 taxa are normal inhabitants of the human mouth (1). Remarkably, these potentially overwhelming populations coexist with the host, with harmful effects occurring only if the immune status is altered or there is a loss of control of epithelial cell sensing and discriminatory systems. It is now generally accepted that this endogenous microflora possesses immunomodulating capacities. Furthermore, some resident commensal bacteria have been shown to provide significant benefit to the host by blocking pathogen colonization and by influencing the normal development of cell structure and the immune system (5, 19, 29). This concept of beneficial bacteria has led to the advent of probiotics, the administration of viable microorganisms that confer health benefits to the host. Manipulation of the resident microflora using probiotics has become a realistic

therapeutic and prophylactic strategy for many inflammatory diseases and infections (39). For example, certain strains of lactobacilli have been suggested to improve mucosal immunity as well as reduce the symptoms caused by a range of conditions, including *Helicobacter pylori* infections, cancer, and inflammatory bowel diseases (12). Clinical trials with humans and animal studies have demonstrated the probiotic properties of commensal bacteria in the oral cavity in the reduction of dental caries, otitis media, and *Streptococcus pyogenes* infection (47).

Certain fundamental questions emerge when considering interactions between epithelial tissues and commensal microbial populations. A major issue concerns the ability of epithelial cells to distinguish between nonpathogenic and pathogenic stimuli, ensuring that resident bacteria do not elicit harmful inflammatory responses, while the host maintains efficient host defenses against pathogens. Some studies have indicated that pathogenic and nonpathogenic bacteria initiate different intracellular signaling pathways and innate immune responses in epithelial cells (7, 16, 27, 35). The mechanisms that allow commensal organisms to be tolerated by epithelial tissues are imperfectly understood, and their dissection has only recently begun, mainly through the study of interactions at the intestinal barrier. A number of studies have suggested that tolerance largely involves specific, active processes causing a functional

modulation of immunity. Some implicate an alteration in Toll-like receptor (TLR) signaling (38), while others have demonstrated suppression by commensals of inflammatory responses in epithelial cells through the inhibition of the NF-κB pathway (8, 24, 34, 48) or through the secretion of IL-10 cytokines (13). Less well studied are the mechanisms by which some commensal organisms are probiotic in function, contributing in additional beneficial ways to epithelial cell function and host-microbe homeostasis.

We aimed to further understand the behavior of epithelial cells in response to the commercially developed probiotic bacterium *Streptococcus salivarius* K12 (20). This commensal bacterium is one of the earliest colonizers of epithelial surfaces in the human mouth and nasopharynx. It is reported to be protective against pathogens causing throat infections, otitis media, pouchitis (47), and oral malodor (4). The protective effects of *S. salivarius* K12 are related in part to the production of salivaricin A2 and salivaricin B, two lantibiotics (antimicrobial peptides) with inhibitory activities toward most *Streptococcus pyogenes* strains (22). Nevertheless, the host response to *S. salivarius* K12, which might contribute to its commensal or probiotic properties, has not yet been studied. To this end, we examined the ability of *S. salivarius* K12 to modulate human epithelial cell immune responses. Through microarray-based analyses and enzyme-linked immunosorbent assay (ELISA), we examined the responses of human bronchial epithelial cells to *S. salivarius* K12 and compared these to the responses elicited by other gram-positive and gram-negative organisms, including opportunists and pathogens. We found that *S. salivarius* K12 downregulated inflammatory responses by inhibiting the NF-κB pathway, actively stimulated beneficial pathways, including type I and II interferon responses, and exerted significant effects on the cytoskeleton and adhesive properties of the host cell. Taken together, these data provide a better understanding of how this probiotic commensal bacterium is tolerated by epithelial cells and contributes actively to the host defense process.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. salivarius* K12 was isolated from BLIS probiotic lozenges (BLIS Technologies, Dunedin, New Zealand) by crushing them into Todd-Hewitt broth (THB) (BD Difco, Franklin Lakes, NJ). Serial dilutions were inoculated onto Mitis salivarius agar (BD Difco, Franklin Lakes, NJ) and incubated at 37°C in a 5% CO_2 atmosphere. Pure cultures were stored at −80°C in dimethyl sulfoxide (7%, vol/vol), and experimental cultures were routinely maintained by weekly passage on Mitis salivarius agar at 37°C in 5% CO_2. For all experiments, broth cultures of *S. salivarius* K12 were generated by inoculating fresh THB with an overnight THB culture (diluted 1:10) and incubating them at 37°C in 5% CO_2 until the mid-exponential phase of growth. *Pseudomonas aeruginosa* strains PAO1, PAK, and PAK's *fliC-* flagellin-negative mutant were grown overnight in Luria-Bertani (LB) agar (BD Difco, Franklin Lakes, NJ) at 37°C, inoculated (1:10) in BM2-glucose minimal medium containing 20 mM Mg^{2+}, and grown until mid-exponential phase at 37°C for further experiments. For microarray experiments, *Salmonella enterica* subsp. *enterica* serovar Typhimurium SL1344 and *Staphylococcus aureus* ATCC 29213 were grown overnight at 37°C on Mueller-Hinton broth.

Biological reagents. The human cationic peptide LL-37 (LLGDFFRKSKEK IGKEFKRIVQRIKDFFRNLVPRTES), was synthesized using F-moc chemistry at the Nucleic Acid/Protein Synthesis Unit, UBC, and purified by high-performance liquid chromatography. The synthetic peptide was resuspended in endotoxin-free water and stored at −20°C until further use. Lipopolysaccharide (LPS) from overnight broth cultures of *P. aeruginosa* H103 was highly purified free of proteins and lipids as described previously (9). Isolated LPS pellets were extracted with a 2:1 chloroform-methanol solution to remove contaminating lipids.

Purified LPS samples were quantified using an assay for the core sugar 2-keto-3-deoxyoctosonic acid (KDO assay) and then resuspended in endotoxin-free water (Sigma-Aldrich, St. Louis, MO). Flagellin was purchased from Invivogen (San Diego, CA) and stored as recommended by the manufacturer. The inhibitor Bay 11-7085 was purchased from Biomol International (Plymouth Meeting, PA).

Tissue culture and coincubation conditions. The simian virus 40-transformed, immortalized human bronchial epithelial cell line 16HBE14O- was a gift from D. Gruenert (University of California, San Francisco). Cells were grown in cell culture flasks (Costar, Cambridge, MA) at 37°C in a 5% CO_2 atmosphere in minimal essential medium (MEM) with Earle's salts (Invitrogen, Burlington, Canada) containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine (complete MEM). They were passaged by treating the monolayer with trypsin-EDTA (Invitrogen, Burlington, Canada) at 37°C for 5 min to dissociate the cells from the flask. Detached cells were transferred to a 50-ml centrifuge tube containing 20 ml complete MEM and then centrifuged for 5 min at $1,000 \times g$. The supernatant was discarded, and cells were resuspended in complete MEM. A 75-cm² flask was seeded with 5×10^5 viable cells in 25 ml complete MEM and incubated at 37°C in 5% CO_2. Cells were used at passage numbers between 6 and 15.

To generate confluent monolayer cultures for experiments investigating interleukin 8 (IL-8) and growth-regulated oncogene alpha (Groα) secretion, 16HBE14O- cells were seeded in 24-well plates (Sarstedt, Newton, NC) at a density of 1×10^5 cells/well. They were grown for 48 h at 37°C in 5% CO_2 in complete MEM. Confluent cells were then coincubated with LL-37 (25 μg/ml and 40 μg/ml) with or without *S. salivarius* K12, *P. aeruginosa* PAO1 with or without *S. salivarius* K12, and flagellin with or without *S. salivarius* K12 (premixed prior to being added to the cells). Mid-exponential-phase broth cultures of bacteria were used at multiplicities of infection (MOI) of 50 viable bacteria per cell, and incubation was in MEM with Earle's salts containing 2 mM L-glutamine but without FBS. The NF-κB inhibitor Bay 11-7085 (40 μM) was added to the epithelial cells 30 min prior to the addition of flagellin and then incubated for 24 h. Supernatants were collected after coincubation for up to 48 h at 37°C in 5% CO_2. They were centrifuged at $10,000 \times g$ for 2 min to remove bacteria and cell debris and stored at −20°C until required.

To generate polarized cell cultures, 16HBE14O- cells were seeded in six-well permeable Transwell plates (Corning Life Science, Corning, NY) at a density of 1.5×10^5 to 3.75×10^5 cells/well. Cells were grown in complete MEM plus penicillin-streptomycin (50 units/ml) (Invitrogen, Burlington, Canada) at 37°C in 5% CO_2 until they were polarized (14 days). Polarization was monitored by measuring the trans-epithelial cell resistance with a Millicell electrical resistance system (Millipore, Billerica, MA). Medium was replaced every other day with fresh medium, and the night before an experiment, it was replaced with fresh complete MEM without antibiotics.

The release of IL-8 induced by *P. aeruginosa* or flagellin in the presence or absence of *S. salivarius* was assessed using polarized 16HBE14O- cells. The medium was replaced with MEM with Earle's salts containing 2 mM L-glutamine but without FBS. *P. aeruginosa*, with or without being premixed with *S. salivarius* (both at an MOI of 50), was applied to the apical surfaces of the polarized 16HBE14O- cells, and supernatants were collected following coculture at 37°C in a 5% CO_2 atmosphere for 6 h. Flagellin (0.5 μg/ml and 1 μg/ml) was added to the apical surfaces of 16HBE14O- cells that had already been cocultured with *S. salivarius* (MOI, 50) for 2 h. Following the addition of flagellin, the cells were further incubated for 5 h and supernatants were collected, centrifuged at $10,000 \times g$ for 2 min, and stored at −20°C until required.

For experiments to determine the global gene responses of epithelial cells, mid-exponential-phase broth cultures of bacteria were added to the apical surfaces of polarized cells at an MOI of 50 viable bacteria per cell. 16HBE14O-epithelial cells and bacteria were incubated together for 1 h in complete MEM. The supernatants containing the bacteria were then replaced by fresh sterile medium, and cells were incubated for a further 3 h, after which they were lysed with RLT lysis buffer supplemented with β-mercaptoethanol (RNeasy mini kit, with RNase-Free DNase treatment [Qiagen, Hilden, Germany]). Lysates were stored at −80°C until extraction of the RNA was performed.

Clonetics primary normal human bronchial epithelial (pnHBE) cells were purchased from Cambrex BioScience Inc. (Walkersville, MD) and were cultured and maintained in bronchial epithelial growth medium (BEGM; Cambrex Bio-Science Inc.), according to the manufacturer's instructions. BEGM is a basal medium (Cambrex BioScience Ltd.) supplemented with bronchial epithelial cell SingleQuots growth factors and supplements (Cambrex BioScience Ltd.) and is used as a serum substitute optimized for the growth and appropriate differentiation of these primary cells. SingleQuots includes human epidermal growth factor, triiodothyronine, bovine pituitary extract, epinephrine, transferrin, insulin, hydrocortisone, gentamicin-amphotericin, and retinoic acid. In accordance

with the manufacturer's instructions, cells were cultured in complete BEGM to 85 to 90% confluence in 100% humidity and 5% CO_2 at 37°C and were used between passages 2 and 3.

Normal primary adult keratinocytes were obtained from Cascade Biologics (Portland, OR). Cells were maintained in Epilife medium with 0.65 μM calcium (Cascade Biologics, Portland, OR) supplemented with the human keratinocyte growth supplement (Cascade Biologics) (contains bovine pituitary extract, bovine insulin, hydrocortisone, bovine transferrin, and human epidermal growth factor) at 37°C in a 5% CO_2 atmosphere according to the manufacturer's instructions and were used between passages 2 and 6. Primary keratinocytes were seeded into tissue culture-treated 24-well plates (Corning Life Sciences, Acton, MA) at a density of 7,000 cells per cm^2 and were cultivated in supplemented EpiLife medium until they attained the desired level of confluence.

RNA extraction, amplification, and hybridization to DNA microarrays. RNA was extracted from 16HBE14O- cells using an RNeasy Mini kit, treated with RNase-free DNase (Qiagen, Hilden, Germany), and eluted in RNase-free water (Ambion, Austin, TX) according to the manufacturers' instructions. RNA was then processed as previously described by Mookherjee et al. (33). Briefly, RNA concentration, integrity, and purity were assessed with an Agilent 2100 Bioanalyzer using RNA 6000 Nano kits (Agilent Technologies, Palo Alto, CA). RNA was (reverse) transcribed with the incorporation of amino-allyl-UTP using the MessageAmpII amplification kit (Ambion, Austin, TX) according to the manufacturer's instructions, and then column purified and eluted in nuclease-free water. Column-purified samples were labeled with the monofunctional dyes cyanine-3 and cyanine-5 (Amersham Biosciences) according to the manufacturer's instructions and then purified using the Mega Clear kit (Ambion, Austin, TX). Yield and fluorophore incorporation were measured using a λ 35 UV–visible-light fluorometer (PerkinElmer Life and Analytical Sciences, Wellesley, MA).

Microarray slides were printed with the human genome 21K Array-Ready oligonucleotide set (Qiagen, Hilden, Germany) at the Jack Bell Research Center (Vancouver, BC, Canada). Slides were prehybridized for 45 min at 48°C in prehybridization buffer containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; Ambion, Austin, TX), 0.1% (wt/vol) sodium dodecyl sulfate (SDS), and 0.2% (wt/vol) bovine serum albumin. Equivalent amounts (20 pmol) of cyanine-labeled samples from control and treated cells were then mixed and hybridized on the array slides in Ambion SlideHyb buffer 2 (Ambion, Austin, TX) for 18 h at 37°C in a hybridization oven. Following hybridization, the slides were washed twice in 1× SSC–0.1% SDS for 5 min at 65°C and then twice in 1× SSC and 0.1× SSC for 3 min each at 42°C. Slides were centrifuged for 5 min at 1,000 × g, dried, and scanned using the ScanArray Express software and scanner (scanner and software by Packard BioScience [PerkinElmer] BioChip Technologies, Wellesley, MA), and the images were quantified using ImaGene (Bio-Discovery, El Segundo, CA).

Analysis of DNA microarrays. Microarray analysis was performed utilizing results from four biological repeats for *S. salivarius* K12, *S. aureus*, and *P. aeruginosa* (two technical repeats per each biological assay) and from five biological repeats for *Salmonella* serovar Typhimirium. Assessment of slide quality, normalization, detection of differential gene expression, and statistical analysis were conducted with the Web-based analysis tool ArrayPipe (18) (www.pathogenomics.ca/arraypipe/) as previously described by Mookherjee et al. (33). Differentially expressed genes were then analyzed using InnateDB, a highly curated database containing biomolecules and their interactions (http://www.innatedb.com) (28b), PANTHER (30), and oPOSSUM (21). Genes that were differentially expressed in response to all treatments were clustered manually, with each cluster representing a set of genes with a distinct pattern of response to a group of treatments, indicating potential coregulation.

qPCR validation. Differential gene expression identified by microarray analysis was validated by quantitative real-time PCR (qPCR) as previously described by Mookherjee et al. (33). The SuperScript III Platinum two-step quantitative reverse transcription-PCR kit with Sybr green (Invitrogen, Burlington, Canada) was used according to the manufacturer's directions in the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). Briefly, 1 μg of total RNA was reverse transcribed in a 20-μl reaction volume for 50 min at 42°C, and the reaction was terminated by incubating the mixture for 5 min at 85°C; then the RNA was digested for 30 min at 37°C with RNase H. The PCR was conducted in a 12.5-μl reaction volume containing 2.5 μl of the 1/10-diluted cDNA template. A melting curve was performed to ensure that any product detected was specific to the desired amplicon. Changes (*n*-fold) were calculated after normalization to endogenous GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and by using the comparative threshold cycle method. The quantitative reverse transcription-PCR primers are included in Table 1.

TABLE 1. Sequences of primers used for qPCR

Gene product	Forward primer (5′–3′)	Reverse primer (3′–5′)
HAMP	CATGTTCCAGAG GCGAAGGA	ACGTCTTGCAGCACA TCCC
TRIP4	GGTTGACCACAC AGGTGCAG	GATCCTGGATTCGCAA CTGG
LNX	TCTAAAGGTCAAC GGGATGGA	CACGCATCACAGTCA GCCAC
PKIG	GATGCGACAGGC ATGATGG	GAGTCTCCCTGGATGT CAGGG
ELMO1	ACACGTGTGGCC GTTTACG	TGGCCACCTTGACGA TGTC
FRDA	CCATCCAGTGGAC CTAAGCG	TTAGTGAGCTCTGCG GCCA
DUSP14	CCAGCACTGCTCA CTTAGGACTT	CCTCCACCAAGGAATC CAAA
GAPDH	GAAACTGTGGCG TGATGG	GTCGCTGTTGAAGTCA GAGG
IL-8	GACCACACTGCG CCAACAC	CTTCTCCACAACCCTC TGCAC
IL-6	AATTCGGTACATC CTCGACGG	GGTTGTTTTCTGCCAG TGCC
RBP1	TGGAAGGTGTGG TCTGCAAG	GGGATCCTGGCTCAC ACATC
IL1RL1	TGGAAGGTGTGG TCTGCAAG	GGGATCCTGGCTCAC ACATC

ELISA assays for IL-8 and Groα detection. IL-8 and Groα secretion were detected using commercially available ELISA kits (BioSource International, Montreal, Canada, and eBioscience, San Diego, CA, respectively) as per the manufacturers' directions. All assays were performed in triplicate. The concentration of IL-8 or Groα in the culture medium was determined by establishing standard curves with serial dilutions of recombinant human IL-8 or Groα. To determine if there was any extracellular breakdown of IL-8 mediated by *S. salivarius* K12, IL-8 (0 to 500 pg/ml culture medium) was incubated with the bacteria (1.0×10^6 to 1.5×10^7 CFU/ml) for 6 h, and the amount of the remaining IL-8 was determined by ELISA.

Western immunoblotting. Cytoplasmic and nuclear proteins were extracted and immunoblotted to test for nuclear translocation of p65 NF-κB as described previously (33). Briefly, pnHBE cells were seeded at 3,500 cells/cm^2 into 60- by 15-mm petri dishes (VWR International, West Chester, PA) and were grown to confluence, and BEBM was changed every other day. When confluent, cells were stimulated for 30 min with flagellin (1 μg/ml) in the presence and absence of *S. salivarius* K12 (MOI, 50:1). Cells were detached by incubation with 0.025% (wt/vol) trypsin–0.01% EDTA, followed by washing and resuspension in ice-cold phosphate-buffered saline. Nuclear and cytoplasmic proteins were extracted using the NE-PER nuclear- and cytoplasmic-extraction reagent kit (Pierce, Rockford, IL) according to the manufacturer's instructions and were stored at −80°C. Following protein determination using the bicinchoninic acid assay (Pierce, Rockford, IL), 3 μg of extracts was resolved by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to immunoblot polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). Membranes were probed with antibodies specific either to the p65 subunits of NF-κB (Cell Signaling Technology, Danvers, MA) or to the H2AX histone (R&D Systems, Minneapolis, MN) as the loading control. Primary antibodies were diluted according to the manufacturer's instructions in TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.01%, vol/vol, Tween 20) containing 5% (wt/vol) skim milk powder. Following reaction with horseradish peroxidase (HRP)-conjugated secondary antibodies, anti-rabbit–HRP antibody for the detection of p65 (Cell Signaling Technology Inc., Danvers, MA), or anti-mouse–HRP antibody for the detection of H2AX histone (Amersham, Piscataway, NJ), membranes were developed using a chemiluminescence peroxidase substrate (Sigma Aldrich) according to the manufacturer's instructions.

LDH assay for cytotoxicity estimation. The levels of lactate dehydrogenase (LDH) in supernatants were assayed in triplicate using a colorimetric cytotoxicity detection kit (Roche, Mannheim, Germany). As a positive control for maximum LDH release, cells were treated with 1% Triton X-100 (Sigma, Oakville, Canada), resulting in complete cell lysis, while LDH release in nontreated cells was used as a negative control. Medium alone was used to assess background (0%).
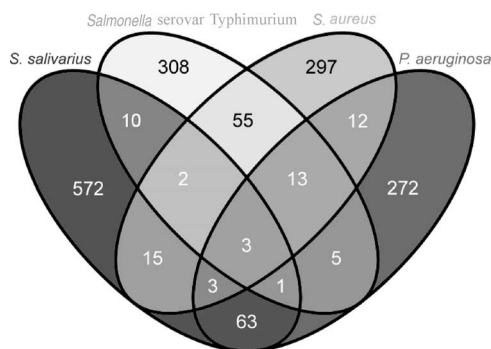
FIG. 1. Venn diagram illustrating the overlap of differentially expressed genes across the four conditions studied.

The percent cytotoxicity was calculated as follows: (experimental value − negative-control value)/(positive-control value − negative-control value) × 100.

Growth of *S. salivarius* with 16HBE14O- cells. To determine the growth properties of *S. salivarius* on the epithelial cell surface, 16HBE14O- cells were seeded in 96-well plates (Sarstedt, Newton, MA) at 1×10^4 cells/well. They were grown for 48 h in complete MEM at 37°C in 5% CO_2. *S. salivarius* bacteria grown in THB until mid-exponential phase were then added to the cells at an MOI of 50. Cells and bacteria were cocultured at 37°C in 5% CO_2 and MEM with Earle's salts containing 2 mM L-glutamine but without FBS. Bacterial growth was monitored by measuring the optical density at 600 nm using a plate reader (Tecan, Salzburg, Austria).

Statistical analysis. ELISA and qPCR results were expressed as means ± standard errors of the means. The two-tail-distribution unpaired Student *t* test was used, and *P* values are indicated in Results. For the recalculation of pathway overrepresentation, immune-related pathways with *P* values above 0.05 were selected, and reviews providing more-complete schematics of these pathways were identified from the literature. Using these more-complete lists of the genes/proteins involved in a pathway rather than the original PANTHER lists, significance values were recalculated using the same binomial test procedure implemented by PANTHER.

Microarray data accession number. Raw microarray data have been deposited in ArrayExpress under accession number E-FPMI-13.

RESULTS

Global array analysis. We examined the effects of *S. salivarius* K12 on global gene expression in 16HBE14O- cells. To highlight the responses that were specific to this organism, and therefore potentially important in its commensal and probiotic activities, we compared gene responses to *S. salivarius* with those initiated by other gram-positive and gram-negative pathogens and opportunistic pathogens.

Polarized 16HBE14O- cells were stimulated with *S. salivarius* K12, *S. aureus*, *P. aeruginosa*, or *Salmonella* serovar Typhimurium for 1 h. Over this period, *S. salivarius* K12 did not grow significantly but adhered efficiently to the epithelial cells, and many remained associated with the cells following stringent washing with phosphate-buffered saline (data not shown). Extracted RNA was used to probe human 21K oligonucleotide-based DNA microarrays. Statistically significantly differentially expressed genes, with a change of at least 1.5-fold and a Student *t* test *P* value of ≤0.06, were extracted using the Web-based analysis tool ArrayPipe (18).

A total of 1,530 unique genes were differentially expressed under one or more conditions. A list of these genes, including accession numbers, descriptions, levels of change, and *P* values, is provided in the supplemental material. VENNY (36) was used to create a Venn diagram (Fig. 1) illustrating the

relationship between genes differentially expressed across each condition. Incubation in the presence of *S. salivarius* led to the differential expression of 660 genes, and *Salmonella* serovar Typhimurium, *S. aureus*, and *P. aeruginosa* caused differential expression of 397, 323, and 367 genes, respectively. *S. salivarius*-stimulated 16HBE14O- cells showed an approximately 5:2 ratio of upregulated to downregulated genes, whereas under the other three conditions, the number of downregulated genes greatly exceeded the number of those upregulated. Interestingly, the response to *S. salivarius* was most similar to the response to *P. aeruginosa*, with 60 genes in common (versus 11 and 16 for *Salmonella* and *Staphylococcus*, respectively).

Clustering of genes. The 1,530 genes were then clustered manually based on their changes in expression across each condition. This resulted in 14 clusters. Clusters 1 through 8 contained genes differentially expressed under only one of the four conditions: clusters 1, 3, 5, and 7 contained upregulated genes, and clusters 2, 4, 6, and 8 contained downregulated genes in response to *S. salivarius*, *Salmonella* serovar Typhimurium, *S. aureus*, and *P. aeruginosa*, respectively. Clusters 9 to 14 contained genes that were up- or downregulated under two or more conditions. Each of clusters 9 to 15 was further divided into subclusters (9.1 and 9.2, etc.). Details of the clustering data and the cluster to which each gene belongs are found in the last column of the supplemental material.

Validation of array gene expression analysis by qPCR. To confirm the results of the array gene expression analysis, genes with significant differential expression in response to *S. salivarius* were selected for validation. Furthermore, to confirm the specific aspect of the clustering analysis, we compared the validated results to results obtained with *P. aeruginosa* (Table 2). We confirmed the expression data of genes with roles that seemed to be particularly relevant in the context of host-microbe interactions (iron and isoprenoid metabolism, cytoskeletal remodeling, receptor signaling and protein kinase activities, and immune function). We also validated the expression data of IL-6 and IL-8 proinflammatory cytokines. As indicated by the array analysis, *P. aeruginosa* was able to activate the gene expression of IL-6 and IL-8, whereas *S. salivarius* K12 did not influence the expression of these genes.

Overrepresented function and process analysis of differentially expressed genes. Differentially expressed genes were next examined in the contexts of molecular function, biological process, and pathway membership. Each of clusters 1 through 8 was subjected to the "compare gene lists" tool at the PANTHER database site (30). The lists were compared to the NCBI *Homo sapiens* genes reference set, without the Bonferroni correction. Functions and processes were noted as overrepresented in a particular cluster if the analysis returned a *P* value equal to or less than 0.01 and if at least two genes annotated with that function/process were found in the cluster. Overrepresented function and process terms are shown in Table 3.

In the *S. salivarius* experiment, uniquely upregulated genes appeared to be involved primarily in homeostatic activities and are perhaps critical to maintaining the commensal host-microbe interaction. Overrepresented homeostatic functions included transcription and translation, protein trafficking, and exocytosis, as well as nucleoside and phosphate metabolism. No such increase in a homeostatic response was evident in

TABLE 2. qPCR validation of array expression data in response to *Streptococcus salivarius* K12 and *Pseudomonas aeruginosa*[a]

Gene name (function)	Organism	qPCR fold change ± SD	P value	Array fold change	P value
HAMP (iron metabolism)	*S. salivarius*	2.0 ± 0.7	<0.01	2.1	0.03
	P. aeruginosa	1.0 ± 0.6	0.68	1.1	0.9
FRDA (iron metabolism)	*S. salivarius*	2.0 ± 0.5	<0.01	3.1	0.05
	P. aeruginosa	1 ± 0.5	0.35	−1.2	0.34
PKIG (protein kinase activity)	*S. salivarius*	2.3 ± 0.8	0.02	4.8	<0.01
	P. aeruginosa	1.4 ± 0.6	0.11	1.2	0.19
DUSP14 (protein kinase activity)	*S. salivarius*	2.1 ± 0.4	0.03	6.4	0.01
	P. aeruginosa	0.9 ± 0.3	0.07	1.9	0.02
TRIP4 (immune activities)	*S. salivarius*	3 ± 1.0	<0.01	2.0	0.00
	P. aeruginosa	1.1 ± 0.5	0.81	1.1	0.3
IL1RL1 (receptor signaling activity)	*S. salivarius*	2.0 ± 0.6	<0.01	2.1	0.05
	P. aeruginosa	1.1 ± 0.2	0.69	1.1	0.64
RBP1 (isoprenoid metabolism)	*S. salivarius*	3.4 ± 0.3	<0.01	6.3	0.01
	P. aeruginosa	1.3 ± 0.4	0.46	1.7	0.09
TMOD4 (actin filament-based process)	*S. salivarius*	2.2 ± 0.6	<0.01	7.6	0.02
	P. aeruginosa	1.5 ± 0.7	0.96	1.8	0.17
IL-8 (inflammation)	*S. salivarius*	1.1 ± 0.2	0.75	1.6	0.07
	P. aeruginosa	3.0 ± 1.2	<0.01	4.6	<0.01
IL-6 (inflammation)	*S. salivarius*	1.2 ± 0.3	0.96	−1.1	0.89
	P. aeruginosa	2.4 ± 0.9	<0.01	2.3	<0.01

[a] Genes with significant differential expression in response to *S. salivarius* were chosen for validation by qRT-PCR. These data were compared with gene expression in response to *P. aeruginosa*. The qPCR and array data presented are the results of a minimum of three biological repeats and two technical repeats. A two-tail-distribution unpaired Student *t* test was performed; the *P* values for the array and qPCR analysis are indicated.

responses to the other three bacteria studied. A large number of transcription factors were also upregulated by the commensal bacterium. Downregulated genes included several genes implicated in cytoskeleton-related functions. Together with the upregulated cytoskeleton-related genes in cluster 1, this may indicate a remodeling of the epithelium in response to *S. salivarius* that promotes a commensal interaction at the epithelial surface. Upon closer examination, a downregulation of several adhesin genes was noted in both the *Salmonella* serovar Typhimurium and *P. aeruginosa* experiments, which may be related to a sloughing response in which the epithelium is destabilized in order to shed pathogenic bacteria.

Because overrepresentation analysis may miss important trends, molecular functions and biological processes were examined more closely. A high-level functional annotation was assigned to each gene by manually inspecting its PANTHER ontologies. For example, genes directly annotated with adhesion ontologies as well as those with adhesion-like ontologies (e.g., tight junction, extracellular matrix, and cadherin) were combined into a larger group annotated simply as "adhesion." When functions were examined from this broader perspective, several interesting trends emerged.

One hundred two genes were noted as having an adhesion-related function. In cells exposed to *S. salivarius*, 45 of these genes were differentially expressed: 23 were upregulated and 12 were downregulated. In the other three organisms, however, the majority of adhesion genes were downregulated (28/37 in

Salmonella serovar Typhimurium, 21/24 in *S. aureus*, and 16/23 in *P. aeruginosa*). A similar trend was observed when transcription factors were examined. In *S. salivarius*, 44 transcription factors were upregulated versus nine that were downregulated. In the other three organisms, the numbers of up- and downregulated transcription factors were roughly equivalent (15 up/16 down, 16 up/14 down, 15 up/15 down in *Salmonella* serovar Typhimurium, *S. aureus*, and *P. aeruginosa*, respectively). Finally, when cytoskeletal and cytoskeleton-related ontologies (e.g., actin, microtubule, and cell structure, etc.) were grouped together, *S. salivarius* exposure led to the upregulation of 29 cytoskeletal genes and the downregulation of only 14, and *P. aeruginosa* exposure led to the up- and downregulation of 12 and 6 genes, respectively. *Salmonella* serovar Typhimurium and *S. aureus*, however, showed markedly different patterns (14 up/13 down and 9 up/10 down, respectively).

Overrepresented-pathway analysis of differentially expressed genes. A similar analysis was then performed using the PANTHER pathway ontology; however, the significance threshold was raised to a *P* of ≤0.05. The only pathway overrepresented in upregulated *S. salivarius* genes was the nicotinic acetylcholine signaling pathway, which was, interestingly, overrepresented in downregulated *P. aeruginosa* genes. Downregulated *S. salivarius* genes were also enriched for the proapoptotic Fas signaling and transforming growth factor β pathways, all of which suggest an attenuation of inflammation in response to the commensal bacterium.

TABLE 3. Selected overrepresented PANTHER molecular functions and biological processes in clusters 1 to 8[b]

Cluster	Organism and regulation[a]	Molecular function(s)	Biological processes	Pathway(s)
1	*Streptococcus salivarius* ↑	Other G protein modulator, transcription factor, actin-binding cytoskeletal protein, helicase, lyase, other chaperones	mRNA transcription, exocytosis, transport, phosphate metabolism, segment specification, neurotransmitter release, muscle contraction	Nicotinic acetylcholine receptor signaling
2	*Streptococcus salivarius* ↓	Serine/threonine protein kinase, cytoskeletal protein, double-stranded DNA binding protein	Signal transduction, cell structure and motility, cell adhesion, transport, cell proliferation and differentiation, phospholipid metabolism, developmental processes, natural killer cell-mediated immunity	TGF-β signaling, FAS signaling, G protein signaling, presenilin, P53 by glucose deprivation, ATP synthesis
3	*Salmonella* serovar Typhimurium ↑	None at a P of <0.01	Inhibition of apoptosis, developmental processes, cell structure and motility, nitrogen metabolism, cell proliferation and differentiation	Inflammation, apoptosis signaling, Toll receptor signaling, Ras, integrin signaling, insulin/IGF-MAPKK/MAPK, angiotensin II-stimulated signaling, EGF receptor signaling
4	*Salmonella* serovar Typhimurium ↓	Cadherin receptor G protein modulator	Cell adhesion, developmental processes, G protein-mediated signaling, neurogenesis vasoconstriction/dilation	Cadherin signaling, G protein signaling, Wnt signaling, axon guidance mediated by semaphorins, inflammation, Parkinson's disease, histamine H1 receptor-mediated signaling, methylcitrate cycle
5	*Staphylococcus aureus* ↑	None at a P of <0.01	mRNA transcriptional regulation	Ras, oxidative-stress response, EGF receptor signaling, apoptosis signaling, B-cell activation, T-cell activation
6	*Staphylococcus aureus* ↓	Immunoglobulin receptor family, defense/immunity protein, serine protease inhibitor, extracellular matrix, apolipoprotein	Cell surface receptor-mediated signal transduction, neuronal activities	Blood coagulation, lipoate biosynthesis
7	*Pseudomonas aeruginosa* ↑	Receptor, chemokine, cytokine, basic helix-loop-helix transcription factor, interleukin	JNK cascade, macrophage-mediated immunity, NF-κB cascade, apoptosis, cell proliferation and differentiation, cytokine- and chemokine-mediated signaling pathway, calcium-mediated signaling, granulocyte-mediated immunity, T-cell-mediated protein phosphorylation, MAPKKK cascade, homeostasis	Apoptosis signaling, interleukin signaling, oxidative-stress response, blood coagulation, Wnt signaling, angiogenesis, presenilin, PDGF signaling, VEGF signaling
8	*Pseudomonas aeruginosa* ↓	Extracellular matrix, cadherin, guanyl-nucleotide exchange factor, voltage-gated potassium channel, acetylcholine receptor, ion channel	Signal transduction, cell adhesion, developmental processes, transport, neuronal activities	Cadherin signaling, Wnt signaling, nicotinic acetylcholine receptor signaling, muscarinic acetylcholine receptor 2 and 4 signaling, endogenous cannabinoid signaling, 5-hydroxytryptamine biosynthesis, metabotropic glutamate receptor group ii, presenilin, G protein signaling

[a] ↑ signifies that genes in this cluster were upregulated; ↓ signifies that genes in this cluster were downregulated.
[b] EGF, epidermal growth factor; IGF, insulin-like growth factor; JNK, Jun N-terminal kinase; MAPKKK, MAPK kinase kinase; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

In contrast, the genes upregulated by the three pathogenic bacteria all showed an overrepresentation of proapoptotic pathways, inflammatory pathways, the oxidative stress response, and platelet-derived growth factor (PDGF) signaling. The Wnt signaling pathway, which may play a role in cell adhesion, was frequently found among downregulated pathogen genes, which is again consistent with an increase in epithelial sloughing in response to pathogenic organisms.

Immune functions among differentially expressed genes. The unique differentially expressed genes were next compared to the InnateDB nonredundantly curated list of 5,570 genes known to be involved in the immune response. Of the 1,530 unique differentially expressed genes, 495 were noted as playing a role in immunity (see the supplemental material). The proportions of up- and downregulated immune genes under each condition were similar to the proportions among all genes, with the exception of the *P. aeruginosa* stimulation genes, of which more immune genes were upregulated than downregulated.

Notably, no cytokines or chemokines were upregulated in response to *S. salivarius*, although the cytokine receptor CCR1 was upregulated. Uniquely upregulated immune gene products in *S. salivarius*-stimulated 16HBE14O- cells included the alpha-2-macroglobulin homolog A2ML1, which acts as an extracellular protease inhibitor, potentially indicating a proactive response to pathogen-secreted proteases, and whose homolog has also been shown to be important for the transport of cytokines; the apoptotic protection factor BNIP1; the epidermal growth factor receptor, which has been shown to negatively regulate TLR2 expression in epithelial cells, thereby attenuating the immune response (31); the hepcidin antimicrobial peptide (HAMP); the anti-inflammatory alpha interferon IFNA2, as well as IRF9, an alpha interferon-responsive transcription factor important in the antiviral response; LY96, required for TLR4 responsiveness to LPS (44); MAP4K4, a kinase encoded upstream of several pathways, including those of Jun N-terminal protein kinase and extracellular signal-regulated kinase (ERK); MAST2 (MAST205), an inhibitor of NF-κB (51); the transcription factor NFE2 (NRF2), which is known to activate a variety of antioxidant genes (40) (reactive oxygen species are known activators of the proinflammatory NF-κB pathway, and thus NFE2's activity may extend to inhibition of this proinflammatory pathway); NUAK1 (ARK5), protective against apoptosis (46); SKIL (SNON), an enhancer of TGFB1 signaling (41); TNFRSF6B (tumor necrosis factor decoy receptor 3), which protects cells against Fas- and LIGHT-mediated apoptosis (17); and TYK2, required for alpha interferon signaling (32).

Notably, downregulated genes included the immunoglobulin and Fc receptors FCAR and FCRLB; the cytokines and cytokine receptor CXCL14, IL-26, and ILRL1; and TRAF3IP2, a known activator of NF-κB signaling (28).

Genes that were not differentially expressed in response to *S. salivarius* but were altered under two or more of the other conditions were also examined. CFLAR was upregulated in response to both *Salmonella* and *Staphylococcus*; it has been shown to activate the NF-κB pathway (23) and, when overexpressed, leads to the repression of Fas-mediated apoptosis in macrophages and a potential increase in inflammation. CCL20 (MIP3-alpha), a potent dendritic cell chemoattractant, was upregulated in response to the two gram-negative pathogens. The IL-10 receptor IL10RA was downregulated in two of the pathogens, indicating a potential dampening of IL-10's anti-inflammatory effects. Interestingly, all three pathogens caused the downregulation of the gamma interferon receptor IFNGR1, which *Mycobacterium tuberculosis* has previously been shown to downregulate as an immune evasion strategy (45).

Alteration of the interferon signaling pathway. Because many pathway databases such as PANTHER are incomplete, pathway overrepresentation analysis can miss important trends. Many pathways are missing (PANTHER, for example, lists gamma interferon signaling but not alpha or beta interferon), and many are missing key genes/proteins. Therefore, certain pathways that were initially not reported as being significantly overrepresented in our analysis were examined in greater detail to determine whether they were being significantly differentially expressed in response to *S. salivarius*.

Of the pathways examined in greater detail in this fashion, one—the unified interferon signaling pathway (including alpha, beta, and gamma interferon signaling)—jumped from a *P* value of >0.05 to *P* value of 1.49E−05, making it the most significantly overrepresented pathway in the data set.

In this pathway (Fig. 2), type I or type II interferons signal through their receptors via JAK1 and TYK2. STAT1 and -2 are recruited, which together with IRF9, leads to the expression of genes downstream of interferon stimulated response elements. Other branches of the system exist, including (i) a branch that leads from JAK1/TYK2 to the p38 pathway, with MAP3K1, MAP2K3, and MAP2K6 as intermediates, and (ii) a branch in which STAT5 is phosphorylated and binds CRKL, both of which are translocated to the nucleus, where they induce the expression of genes downstream of gamma interferon activated site elements (37). In response to *S. salivarius*, 16HBE14O- cells upregulated the expression of several of these genes (IFNA2, TYK2, MAP3K1, CRKL, IRF9). Furthermore, the p38 pathway activated by type I interferon signaling is known to result in the activation of a number of transcription factors, including CREB, the binding sites for which were significantly overrepresented in *S. salivarius*-responsive genes (see Discussion). Through the p38 pathway, genes with antiviral and cytokine modulation properties are activated (37), while genes regulated by interferon stimulated response element and gamma interferon activated site regions are overwhelmingly skewed toward host defense.

Immediate early gene expression is often controlled by cyclic AMP (cAMP)-dependent protein kinase A (PKA) signaling, which results in the transcription of CREB-responsive genes. *S. salivarius* not only stimulated the expression of several of these CREB-responsive genes but also upregulated the expression of protein kinase inhibitor G, an inhibitor of PKA signaling that can rapidly turn off the expression of these genes (6).

Transcription factor binding site analysis. By examining the transcription factor binding sites present in the upstream regions of the differentially expressed genes, the potential pathways governing these changes in expression could be inferred. Promoter regions of the differentially expressed genes were submitted to oPOSSUM (21), which identifies overrepresented transcription factor binding sites in these regions using the vertebrate profiles from the JASPAR database (3). Default
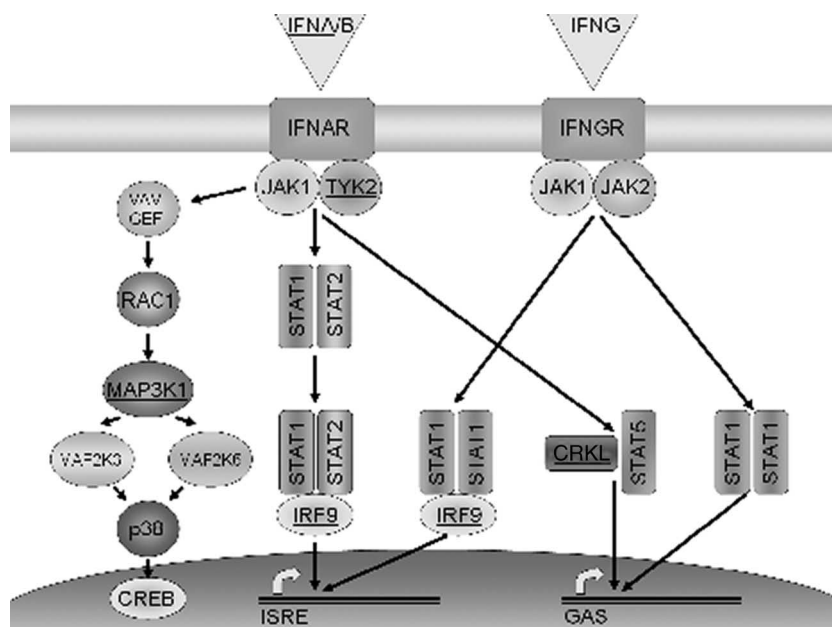
FIG. 2. Impact of *S. salivarius* on interferon signaling pathways. Underlined genes were upregulated by *S. salivarius* K12 alone. IFNA/B, alpha/beta interferon; IFNG, gamma interferon; IFNAR, alpha interferon receptor; IFNGR, gamma interferon receptor; ISRE, interferon stimulated response element; GAS, gamma interferon activated site; GEF, guanine nucleoside exchange factor; CRKL, CRK-like protein.

parameters were used to search the regions 500 bp upstream from the transcription start sites. The top 10 overrepresented transcription factor binding sites according to both the *Z*-score and Fisher score were retrieved, duplicates were removed, and the resulting list is shown in Table 4.

Strikingly, binding sites for the NF-κB family of transcription factors were found to be overrepresented in two of the three pathogen-stimulated gene sets yet not in the genes differentially expressed in response to *S. salivarius*, indicating that pathways converging on NF-κB were either not active or limited in their activity in response to the commensal bacterium, again contributing to the anti-inflammatory properties of *S. salivarius*.

Reduction of baseline IL-8 secretion by *S. salivarius* K12 in polarized 16HBE14O- cells. A number of findings in the microarray analyses pointed strongly toward *S. salivarius* K12 having anti-inflammatory activities, particularly in influencing pathways converging on NF-κB. Therefore, we examined the ability of this organism to modulate the inflammatory response in the human bronchial epithelial cell line 16HBE14O-. IL-8 secretion was analyzed, as this chemokine is one of the major mediators of the inflammatory responses of several cell types, functioning to recruit neutrophils to the site of infection. *S. salivarius* had no cytotoxic effects on 16HBE14O- cells, as indicated by measuring LDH release from cells incubated with the bacteria for up to 48 h (data not shown). *S. salivarius* K12 exerted an anti-inflammatory effect on the epithelium by significantly downregulating the secretion of IL-8. Basal IL-8 secretion of *S. salivarius* K12-infected epithelia was reduced to 40% compared to that of uninfected epithelia after 6 h of incubation (Fig. 3D) and to 55% and 82% of the baseline level after 24 and 48 h of coincubation, respectively (Fig. 3A and B). Coincubation of IL-8 protein with *S. salivarius* K12 indicated

that the bacterium caused no extracellular breakdown of the cytokine (data not shown), confirming that the observed changes in levels of IL-8 are due to the suppression of secretion.

Attenuation by *S. salivarius* of IL-8 secretion induced in 16HBE14O- cells by inflammatory mediators. The ability of *S. salivarius* K12 to attenuate proinflammatory responses induced by factors known to induce IL-8 secretion by epithelial cells, namely, *P. aeruginosa* cells, endotoxin, flagellin, and LL-37, was next examined. Flagellin and LPS did not adversely affect the 16HBE14O- cells, while *P. aeruginosa* and LL-37 exerted some cytotoxic effects, as indicated by measuring LDH release from cells incubated with the bacteria for 6 h or with LL-37 for 24 h (~15% increase over the baseline LDH release) (data not shown).

P. aeruginosa induced the release of IL-8 from polarized 16HBE14O- cells after 6 h of incubation. When *S. salivarius* was also included, IL-8 secretion was significantly attenuated ($P < 0.002$) (Fig. 3D). Bacterial flagellin and LPS have been shown to be especially immunostimulatory, causing the upregulation and secretion of IL-8 through interactions with TLR5 and TLR4, respectively (43). *Salmonella* serovar Typhimurium flagellin (0.5 μg/ml and 1 μg/ml), applied to the apical surfaces of polarized 16HBE14O- cells for 5 h, stimulated IL-8 secretion in a dose-dependent manner compared with what occurred with nonstimulated cells. This secretion was significantly attenuated ($P < 0.001$) in the presence of *S. salivarius* K12 (Fig. 3E); when increases above the basal levels of IL-8 secretion are considered, then K12 reduced *P. aeruginosa*-induced IL-8 secretion by 62% and secretion induced by 1.0 μg/ml flagellin was suppressed by 75%. *P. aeruginosa* LPS did not induce the release of IL-8 from the 16HBE14O- cell line (data not shown), a response typical of epithelial cells.

TABLE 4. Most overrepresented transcription factor binding sites in the promoter regions of the differentially expressed genes[a]

Organism	TFBS	Z-score
S. salivarius	**CREB1**	5.84
	Evi1	18.7
	FOXI1	6.45
	Foxq1	8.35
	MEF2A	5.53
	NHLH1	5.23
	RXR-VDR	9.50
	Sox5	6.47
	SRY	9.29
Salmonella serovar Typhimurium	**E2F1**	7.33
	FOXF2	5.55
	Myf	4.24
	NF-κB	7.10
	NFKB1	5.90
	REL	5.05
	RELA	8.80
	Staf	5.28
S. aureus	Foxd3	7.56
	Gfi	9.463
	HNF1A	9.38
	Lhx3	6.921
	Nkx2-5	11.97
	NKX3-1	5.597
	Prrx2	7.153
	RORA	10.54
	Sox5	8.026
	TBP	7.152
	Foxd3	7.56
P. aeruginosa	Foxa2	9.56
	Foxd3	10.2
	FOXI1	10.3
	Gfi	10.3
	MEF2A	8.26
	NF-κB	3.69
	Nkx2-5	9.26
	Pax4	24.3
	REL	6.30
	RXR-VDR	7.58
	Sox5	8.62
	SRF	6.53
	SRY	9.07

[a] Transcription factor binding sites (TFBS) in bold were unique to that particular experimental condition. NF-κB and its subunits are underlined. A Z-score of greater than 5 is considered to be statistically significant. For *P. aeruginosa*, NF-κB was also included, since an alternative statistical test (Fisher's exact test) suggested a *P* value of 0.036.

Human cathelicidin LL-37, a cationic host defense peptide expressed primarily by neutrophils and epithelial cells, is upregulated under conditions of inflammation and is a known immunomodulatory peptide that leads to the upregulation of chemokines while suppressing the expression of most proinflammatory cytokines (33). When added with *S. salivarius* to 16HBE14O- cell supernatants, LL-37 delayed the exponential-phase growth of the bacteria but did not kill them (Fig. 3C). As previously demonstrated (2), the addition of 25 and 40 μg/ml LL-37 alone to 16HBE14O- cells for 24 and 48 h stimulated IL-8 release from these cells in a dose-dependent manner (Fig. 3A and B). *S. salivarius* significantly attenuated LL-37-mediated IL-8 secretion (Fig. 3). When increases above the basal levels of IL-8 secretion are considered, then IL-8 induction by

25 μg/ml LL37 was reduced by more than 80% after 24 h and 48 h of incubation in the presence of *S. salivarius* K12. Similarly, K12 caused 70% (24 h) and 80% (48 h) suppression of IL-8 secretion above basal levels when cells were stimulated with 40 μg/ml LL37.

Attenuation by *S. salivarius* of IL-8 secretion induced in primary normal human bronchial epithelial cells and primary keratinocytes by *P. aeruginosa*. *P. aeruginosa* PAK stimulated pnHBE cells to secrete 318 pg/ml (over baseline secretion) IL-8. This response was entirely due to flagellin, as a *fliC*-negative mutant strain did not induce IL-8 secretion at all. When *S. salivarius* was also included, IL-8 secretion was reduced to only 5.1 pg/ml (Fig. 4A). This result indicates that *S. salivarius* exerted a considerable anti-inflammatory effect on the pnHBE cells. Similar experiments performed on primary keratinocytes also demonstrated that *S. salivarius* downregulated IL-8 secretion in response to PAK and flagellin (Fig. 4B), further indicating that this phenomenon occurs in multiple epithelial cell types.

Attenuation by *S. salivarius* of 16HBE14O- cell Groα secretion induced by flagellin. Groα (CXCL1) is an inducible neutrophil chemotactic factor synthesized in epithelial tissues during inflammation. This chemokine was first identified as a melanoma growth-stimulatory factor and has further been shown to stimulate a number of biological responses, including chemotaxis, angiogenesis, and growth regulation. Flagellin stimulated the release of Groα from 16HBE14O- cells. Coincubation of 16HBE14O- with flagellin and the NF-κB inhibitor Bay 11-7085 reduced the secretion of Groα to below the background level, demonstrating that Groα production is induced in 16HBE14O- cells in response to flagellin in an NF-κB-dependent manner (Fig. 5). Coincubation of flagellin with *S. salivarius* K12 attenuated Groα secretion by 56%, thus indicating the ability of *S. salivarius* to inhibit the NF-κB pathway (Fig. 5).

***S. salivarius* inhibited NF-κB P65 subunit translocation into the nucleus.** pnHBE14 stimulation with flagellin (1 μg/ml) for 30 min induced the translocation of the P65 subunit of NF-κB into the nucleus, further confirming the proinflammatory effect of this TLR agonist. The addition of *S. salivarius* alone to pnHBE cells resulted in no nuclear translocation of the subunit, and K12 greatly reduced flagellin-induced P65 translocation (Fig. 6). This confirms the ability of *S. salivarius* K12 to inhibit the activation of the NF-κB pathway.

DISCUSSION

The ability of epithelial cells to sense the external environment and communicate this information to the local immune system, thereby initiating appropriate responses, is essential for the maintenance of health and prevention of the development of chronic inflammatory diseases. Our studies have shown that an oral probiotic commensal strain of *S. salivarius* is able to inhibit inflammatory responses in human bronchial epithelial cells by downregulating the NF-κB pathway. This is consistent with an emerging paradigm that indicates the downregulation of epithelial immune responses by commensal bacteria (8, 13, 24, 38, 48). Not only did *S. salivarius* K12 inhibit baseline synthesis of IL-8, but it also suppressed IL-8 secretion when cells were stimulated with pathogenic *P. aeruginosa*, *Sal-*
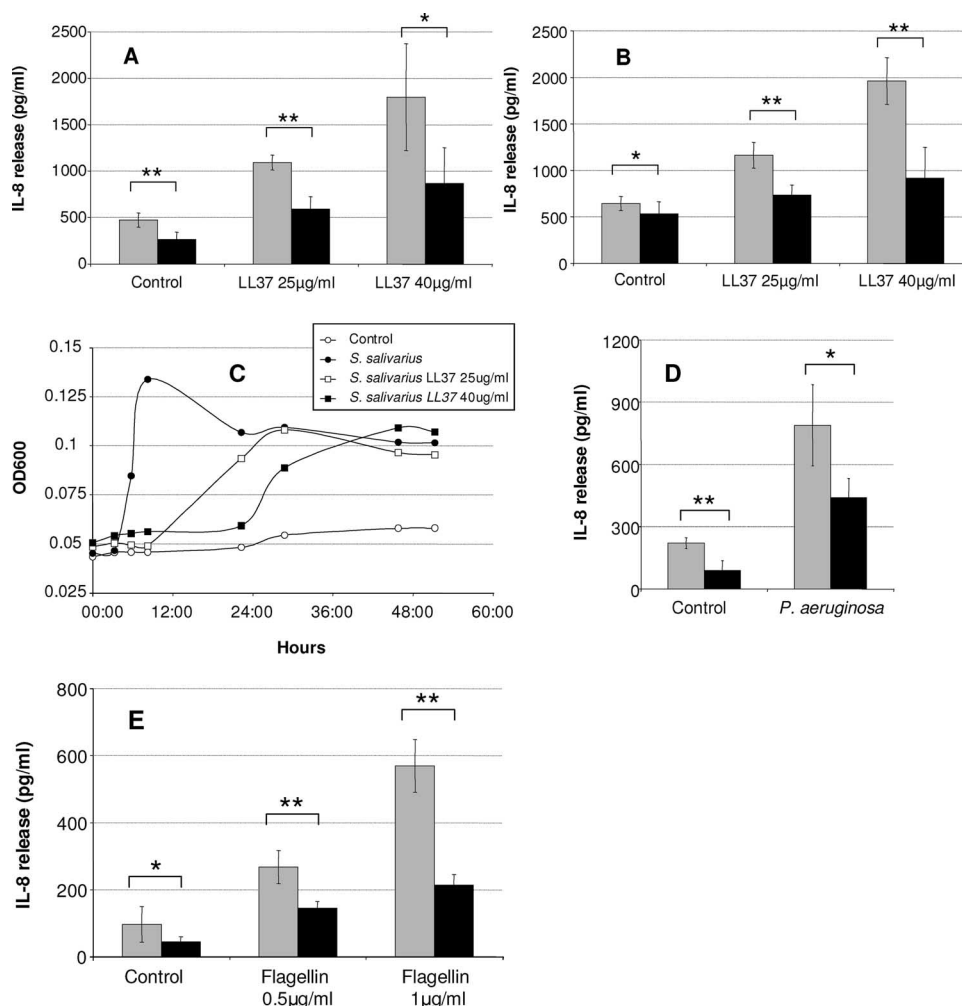
FIG. 3. *Streptococcus salivarius* K12 downregulates IL-8 release from human bronchial epithelial cells (16HBE14O-) in response to LL-37, *Pseudomonas aeruginosa*, endotoxin, and flagellin. IL-8 release from 16HBE14O- cells was monitored after incubation with LL-37 (A and B), with *P. aeruginosa* (D), and with flagellin (E) in the presence (black bars) or absence (gray bars) of *S. salivarius* (MOI, 50). LL-37 was incubated with cells for either 24 h (A) or 48 h (B). Growth properties of *S. salivarius* on 16HBE14O- cells in the presence of LL-37 (25 and 40 μg/ml) was monitored (C). *P. aeruginosa* (MOI, 50) was incubated with cells for 6 h. *S. salivarius* was incubated for 2 h before flagellin (0.5 and 1 μg/ml) was added and further incubated for 5 h (7 h of total incubation). The data are the result of a minimum of three biological repeats and two technical repeats. A two-tail-distribution, unpaired Student *t* test was performed (∗∗, P < 0.01; ∗, P < 0.05). OD600, optical density at 600 nm.
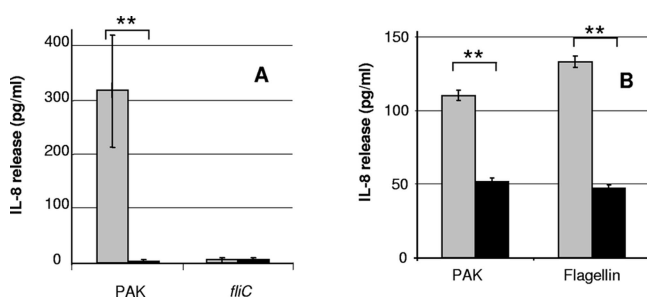

FIG. 4. *Streptococcus salivarius* downregulates IL-8 secretion from primary normal human bronchial epithelial cells and primary keratinocytes. IL-8 release from pnHBE cells (A) or primary keratinocytes (B) was monitored after incubation with *P. aeruginosa* PAK, the PAK *fliC*-negative mutant, or flagellin (1 μg/ml) in the presence (black bars) or absence (gray bars) of *S. salivarius* K12. Conditions were as follows: *S. salivarius* was added to the cells at the same time as *P. aeruginosa* (MOI, 50:1) or flagellin, and the cells were incubated for 6 h. ∗∗, P < 0.01.

monella serovar Typhimurium flagellin, or the immunomodulatory host defense peptide LL-37. Most previous studies have focused on IL-8 and IL-6 responses, but here it was demonstrated that this commensal bacterium was able to attenuate Groα secretion, consistent with the gene expression data of Tien et al. (48). Functional genomic analyses further identified a role for *S. salivarius* K12 in the specific modulation of genes associated with innate response pathways as well as general epithelial cell function and homeostasis. This may help to explain the beneficial probiotic activities of *S. salivarius* K12 and its potential role in the maintenance of host-microbe homeostasis.

The IL-8 secretion pathways activated by the stimuli employed in this study have been characterized. LL-37-induced IL-8 production in 16HBE14O- cells is mediated through the phosphorylation and activation of the mitogen-activated protein kinases (MAPKs) ERK1/2 and p38 (2). *P. aeruginosa* LPS,
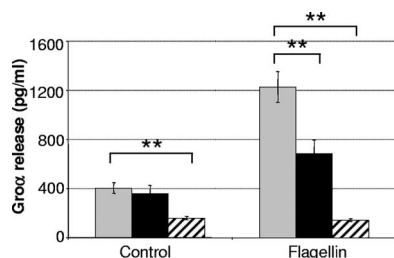
FIG. 5. *Streptococcus salivarius* K12 downregulates Groα release from human bronchial epithelial (16HBE14O-) cells in response to flagellin by inhibiting the NF-κB signaling pathway. Groα release from 16HBE14O- cells was monitored after they were incubated with flagellin (1 μg/ml) and compared with the level in the control. 16HBE14O- cells were incubated with *S. salivarius* (MOI, 50) (black bars), with the NF-κB inhibitor Bay 11-7085 (40 μM) (hatched bars), or nothing (gray bars). Conditions were as follows. *S. salivarius* was added to the epithelial cells 2 h prior to the addition of flagellin (0.5 and 1 μg/ml) and incubated for 24 h (26 h of total incubation). The NF-κB inhibitor Bay 11-7085 (40 μM) was added to the epithelial cells 30 min prior to the addition of flagellin, and then the cells were incubated for 24 h. ∗∗, $P < 0.01$; ∗, $P < 0.05$.

FIG. 6. *S. salivarius* inhibits NF-κB P65 subunit translocation into the nucleus. Western blotting of P65 NF-κB subunits was performed with nuclear extracts of 16HBE14O- cells following their incubation with 1 μg/ml flagellin or *S. salivarius* (MOI, 50:1) or with S. salivarius (MOI, 50:1) plus 1 μg/ml flagellin. Reaction with antibody against histone H2AX was used as a loading control. Results are representative of three independent experiments.

pilin, flagellin, peptidoglycan, and virulence factors have been shown to activate MAP kinases (p38 and ERK1/2) and the NF-κB pathway to induce IL-8 secretion (14, 49, 50, 53). We further showed that flagellin stimulates Groα release from 16HBE14O- cells through NF-κB activation and that this response was attenuated in the presence of *S. salivarius*. Our demonstration (Fig. 6) that *S. salivarius* K12 likely exerted its anti-inflammatory effects through inhibiting NF-κB activation in 16HBE14O- human bronchial epithelial cells is consistent with the results of analogous studies of another commensal in human colon adenocarcinoma cells (HT29) (11).

To emphasize the very different nature of the response elicited by this commensal, we compared the patterns of global transcriptional responses of epithelial cells stimulated with *S. salivarius* with responses elicited by selected gram-positive and gram-negative pathogens. Only a limited number of studies have examined transcriptional responses to commensal organisms (16, 19), but comparisons with pathogens were retrospective rather than done in parallel. Comparison of two analyses by the same group indicated that the commensals *Streptococcus gordonii* and *Fusobacterium nucleatum* (16) promoted a more restrained response than the periodontopathogens *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* (15). However, both *P. gingivalis* and *A. actinomycetemcomitans* (except the JP2 clone) are opportunistic, rather than frank, pathogens in the mouth, as they resemble commensals in their genetic diversity, acquisition, and population structures (25). Unlike with these studies, it was demonstrated here that *S. salivarius* K12 induced widespread and quite different alterations in gene expression from those induced by the tested pathogens and opportunistic pathogens in regulating the expression of 660 genes, of which 565 were specifically regulated by this commensal bacterium. It was observed that larger numbers of *S. salivarius* K12 than of *P. aeruginosa* bacteria remained associated with the 16HBE14O- cells, and this may in part have contributed to the more extensive gene expression changes observed. However, this observation also underlines the noninflammatory na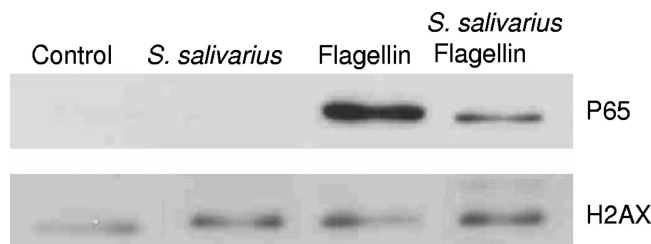ture of the immune response to this commensal organism. This comparative analysis confirmed the immunosuppressive properties of *S. salivarius* K12 toward genes stimulated through the NF-κB signaling pathway, in that NF-κB binding sites were overrepresented in the promoter regions of pathogen-modulated genes but not in those modulated by the commensal *S. salivarius*, even though this commensal contains such TLR agonists as lipoteichoic acid, peptidoglycan, and CpG DNA.

The nicotinic acetylcholine pathway was also overrepresented in *S. salivarius*-treated cells but not in pathogen-treated cells. This pathway has been noted for its anti-inflammatory potential, mediated through the suppression of IκB phosphorylation and subsequent inhibition of NF-κB-induced transcription (52). CREB binding sites were significantly overrepresented in *S. salivarius*-responsive genes but not in pathogen-treated cells; CREB is a transcription factor whose activity has been related to anti-inflammatory properties (10). InnateDB was further used to identify immune functions among differentially expressed genes. *S. salivarius* is able to modulate the expression of regulators central to multiple innate response pathways. Notably, the most broadly affected pathway was the interferon signaling system. The IFN pathway is recognized as a central mediator of the immune response, with pleiotropic properties such as antiviral and antitumor activities, the activation of microbicidal effector functions, leukocyte trafficking, priming of the LPS response, and anti-inflammatory effects (42). Our analyses, consistent with direct measurements of secreted cytokines, also indicated that *S. salivarius* K12 did not initiate the synthesis of proinflammatory cytokines or chemokines, nor did the organism regulate genes involved in responses to such molecules. This absence of induced cytokine host responses to probiotic bacteria was also reported in a study comparing the cytokine expression profiles elicited by the probiotic bacteria *Bifidobacterium infantis* 35624 and *Lactobacillus salivarius* to responses elicited by *Salmonella* serovar Typhimurium UK1 (35).

Other oral commensal strains have been shown to differentially induce or repress cytokine release in oral keratinocytes (16, 26), thus indicating that commensal organisms can have different impacts on the immune responses in host cells. This underlines the complex and dynamic host response that may result from interaction with a complex bacterial community as it occurs in vivo. Our recent data (unpublished) indicate that

many commensal streptococci isolated from the tongue are, like *S. salivarius* K12, capable of suppressing the immune responses of epithelial cells, further emphasizing that this as a significant phenomenon that may contribute to host-microbe homeostasis and that it is not just a property of a few well-studied commensal or probiotic bacteria. The phenomenon is also not unique to the responses of the 16HBE14O- cell line, as we observed similar effects on primary bronchial epithelial cells and primary keratinocytes, and our recent data demonstrated the same effect on the responses of a dysplastic oral keratinocyte cell line derived from the tongue. 16HBE14O- cells therefore represent a well-characterized, consistent, and robust model of the responses of epithelial cells to bacteria that are isolated from a variety of anatomical sites (ranging from the mouth and skin to the respiratory tract and intestine).

In addition to modulating immune and innate defense responses, analysis of gene clusters further identified a role for *S. salivarius* K12 in the specific modulation of genes associated with homeostatic functions such as transcription and translation, protein trafficking and exocytosis, and nucleoside and phosphate metabolism. A significant effect on genes with adhesion and cytoskeleton-related functions was specifically observed in response to *S. salivarius* K12. These observations indicate that this organism exerts a significant effect on the adhesive and structural properties of 16HBE14O- cells, a response that may act to strengthen the interactions between commensals and the host and may help in maintaining tight junctions of cells on epithelial surfaces.

Therefore, we suggest that the commensal and probiotic behaviors of *S. salivarius* K12 may be due to the organism eliciting, in epithelial cells, (i) a response that is not proinflammatory; (ii) the dysregulation of genes involved in signal transduction cascades central to multiple innate response pathways which modulate the general defense response after interacting with microbes and immunostimulatory molecules; (iii) the regulation of genes that mediate a downregulation of the NF-κB pathway, resulting in the attenuation of some innate immunity pathways involved in the proinflammatory responses of epithelial cells; and (iv) the specific modulation of genes associated with general epithelial cell functions and homeostasis and adhesive and structural cellular properties. Through modulation of these physiological responses and innate defenses, we propose that *S. salivarius* K12 ensures not only that it is tolerated by the host but also that it promotes cellular health and homeostasis and may therefore protect host tissues from damage caused by other immunostimulatory cells and products.

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